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13. ABSTRACT (Maximum 200 Words)

Tumor progression induces the growth of endothelial cells by releasing angiogenic factors. This is accompanied by down-regulation of local tissue inhibitors of endothelian cell proliferation such as angiostatin and endostatin. Both proteins target normal endothelial cells and effectively regress large tumors in animals. However, animal studies demonstrate that an effective treatment requires long-term administration of angiogenesis inhibitors. Thus, delivery of angiogenesis inhibitor genes to tumor sites should increase local concentration of these proteins, leading to the retardation of tumor progression and metastasis. During this funding period, we have established stable human prostate cancer cell lines derived from PC3 cells transduced with HIV7/endo, HIV7/angio or both together. Expression of the angiogenesis inhibitors did not affect the proliferation of PC3 cells significantly. These PC cell lines produced and secreted sufficient amounts of angiogenesis inhibitors to inhibit the proliferation of primary human primary human umbilical vein endothelial cells (HUVEC) in culture. Subcutaneous implantation of these cells onto nude mice demonstrates that both proteins exert an effect on the ability of PC3 cells to form tumor *in vivo*.

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Introduction

Tumor progression induces the growth of endothelial cells by releasing angiogenic factors. This is accompanied by down-regulation of local tissue inhibitors of endothelial cell proliferation such as angiostatin and endostatin. Both proteins target normal endothelial cells and effectively regress large tumors in animals. However, animal studies demonstrate that an effective treatment requires long-term administration of angiogenesis inhibitors. Thus, delivery of angiogenesis inhibitor genes to tumor sites should increase local concentration of these proteins, leading to the retardation of tumor progression and metastasis. We propose to use HIV vectors to deliver the endostatin and angiostatin genes into human prostate cancer cell lines in culture. The effect of these two proteins will be evaluated by tumor formation and metastasis in nude mice grafted with the transduced cells. During the entire funding period, we cloned the cDNAs for endostatin and angiostatin into 3rd generation HIV vectors that also contained the gene encoding green fluorescence protein (GFP) for the marking of the transduced cells. We showed that direct delivery of the angiogenesis inhibitor genes into human tumor cell lines, including fibrosarcoma HT1080 cells, immortalized prostate epithelial 267B1/v-Ki-ras cells and prostate cancer PC3 cells, did not affect cell proliferation in culture. In contrast, cell proliferation was strongly inhibited by these proteins in EJG cells derived from bovine adrenal gland capillary endothelial cells and in primary human umbilical vein endothelial cells (HUVECs). Similarly, when the angiogenesis inhibitors secreted from the transduced tumor cells were concentrated, they were capable of inhibiting the proliferation of normal epithelial cells. Further, implanting PC3 prostate cancer cells expressing the angiogenesis inhibitors onto nude mice led to significant slowdown in tumor progression *in vivo*. These results suggest that the proposed approach of delivering angiogenesis inhibitor genes into prostate cancer cells for the production and secretion of these proteins to block angiogenesis surrounding the tumor site may represent a reasonable strategy to fight tumors.

Body

1. Expression of endostatin and angiostatin did not affect cancer cell proliferation in culture. To determine the effect of angiogenesis inhibitors on cell proliferation, the cDNAs for endostatin and angiostatin were cloned into a 3rd generation HIV vector, pHIV7/GFP [1]. This vector also contains the GFP gene for the marking of the transduced cells. As shown in Fig. 1, transduction of fibrosarcoma HT1080 cells with these vectors had little effect on cell proliferation. Although HIV/angio transduction led to decreased cell proliferation at a moi of 2, this effect was probably non-specific since transduction with both vectors at the same moi did not significantly alter cell proliferation (Fig. 1, endo-angio). Transduction of 267B1/v-Ki-ras cells derived from human prostate epithelial cells [2] with HIV7/endo also did not significantly affect cell proliferation in culture when compared with cells transduced with the control vector, HIV/GFP (Fig. 2). At the moi used for such an experiment, approximate 70% of the 267B1/v-Ki-ras cells expressed GFP. Thus, inefficient transduction by HIV/endo could not account for the similar cell proliferation rate in culture. Transduction of prostate cancer PC3 cells with these vectors also demonstrated little effect on cell proliferation (Fig. 3). To demonstrate angiogenesis inhibitor expression, the culture supernatant and cell extract of PC3 cells transduced with either HIV/GFP or HIV/angio were prepared and subjected to Western blot analysis using an antibody specific for the HA tag attached to angiostatin [3]. As shown in Fig. 4, angiostatin expression was

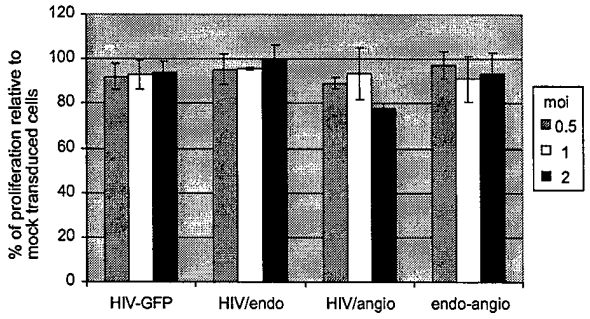

Fig. 1. The effect of HIV/endo and HIV/angio transduction on HT1080 proliferation.

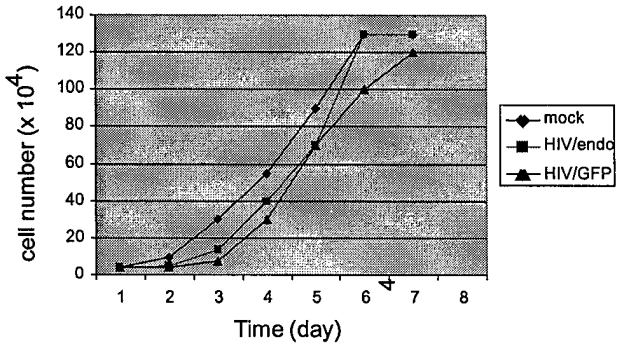

Fig. 2. The effect of endostatin on proliferation of transduced 267B1/v-Ki-ras cells.

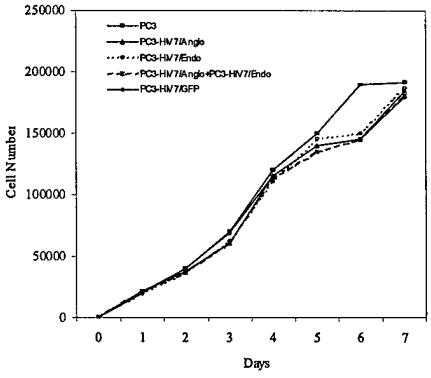

Fig. 3. The growth rate of HIV vector-transduced PC3 cells.

readily detectable in the culture supernatant and the cell extract of the transduced PC3 cells. We were not able to confirm endostatin expression as no antibody specific for this protein was available. These results suggest that angiogenesis inhibitors exert little effect on tumor cell growth.

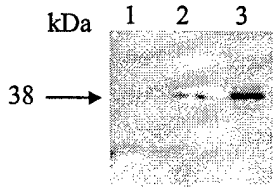

Fig. 4. Western blot analysis of angiostatin expression in HIV7/angio transduced PC3 cells. Lanes 1& 2 contain concentrated culture supernatants from PC3 cells transduced with HIV/GFP and HIV/angio, respectively; lane 3 contains the cell extract from PC3 cells transduced with HIV/angio.

2. Expression of endostatin and angiostatin blocked endothelial cell proliferation.

To determine the effect of endostatin and angiostatin on endothelial cell proliferation, we transduced EJG cells derived from bovine adrenal gland capillary endothelial cells with HIV/endo. As shown in Fig.5, EJG cell proliferation was inhibited approximately 50% by the endostatin vector, but not by the control GFP vector. Similar experiments were performed with primary human umbilical vein endothelial cells (HUVECs) using HIV/endo and HIV/angio. As shown in Fig. 6, HUVEC proliferation was inhibited by the endostatin and angiostatin vectors applied at various moi but not by the GFP control vector. To determine whether the angiogenesis inhibitor generated from transduced cancer cells is functional and can inhibit endothelial cell proliferation, the culture supernatant from mock- or vector-transduced cancer cells was collected and concentrated 5 fold. The concentrated culture medium was applied to EJG cells and cell proliferation was determined. As shown in Fig. 7, proliferation of EJG cells treated with the culture medium from HIV/endo-transduced 267B1/v-Ki-ras cells was reduced relative to that from mock- or HIV/GFP-transduced cells. Similar results were obtained when the culture supernatants from transduced PC3 cells were used to treat HUVECs and cell

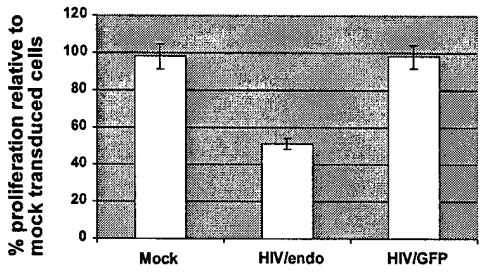

Fig. 5 The effect of HIV/endo transduction on EJG cell proliferation.

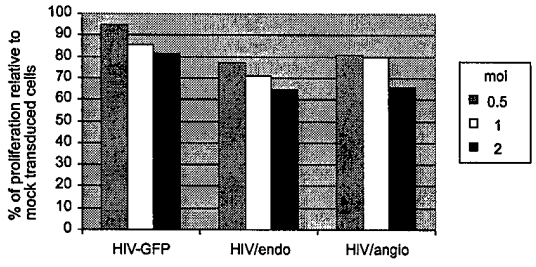

Fig. 6. Expression of endostatin and angiostatin inhibits HUVEC proliferation.

proliferation was assayed (Fig. 8). These results suggest that angiogenesis inhibitors secreted from transduced cancer cells can block endothelial cell proliferation in culture.

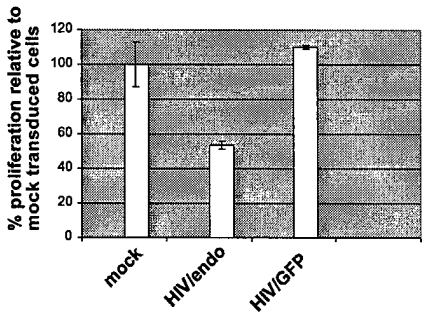

Fig. 7. Inhibiton of EJG cell proliferation with endostatin harvested from the culture media of transduced 267B1/v-Ki-ras cells.

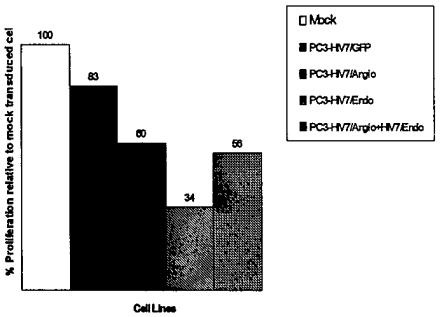

Fig. 8. Inhibition of HUVEC proliferation by the culture supernatant of HIV vector-transduced PC3 cells.

3. Inhibition of tumor formation by angiogenesis inhibitors *in vivo*. To determine the effect of angiogenesis inhibitor expression on tumor formation *in vivo*, PC3 cells were repeatedly transduced with the HIV vector. The cell fraction containing the stably transduced cells was determined by FACS analysis. As shown in Table 1, more than 80% of the PC3 cells were GFP+ with each vector. Since the GFP gene in each vector was linked to the endostatin or the angiostatin cDNA through an internal ribosome entry site (IRES), stable GFP expression served as a good indicator for the expression of the angiogenesis inhibitor as well. Nude mice were separated into five groups with seven mice in each group. Each pooled culture shown in Table 1 was injected subcutaneously at a dose of 10^6 cells/injection site. Tumor progression was followed by size measurement and all animals were sacrificed 6 weeks later. At 5 1/2 week after inoculation, expression of endostatin, angiostatin or a combination of both factors in PC3 cells led to a reduction in tumor size by 67%, 38% and 53%, respectively. In contrast, GFP expression in PC3 cells led to a reduction in tumor size by 28% only. It is interesting that no clear difference was observed at 3 1/2 week after inoculation. As PC3 cells derived from HIV/angio had higher percentage of GFP+ cells when compared with that derived from HIV/endo (Table 1), this result also suggested that endostatin was more robust than angiostatin to block tumor progression *in vivo*. Since co-expression of endostatin and angiostatin in PC3 cells did not render more efficient inhibition of tumor progression when compared with PC3 cells expressing endostatin alone, these two factors may have no additive effect on angiogenesis inhibition.

Table 1. Percentage of GFP+ PC3 cells transduced with different HIV vectors.

mock	HIV/ GFP	HIV/ endo	HIV/ angio	HIV/endo + HIV/angio
0%	95%	84%	95%	80%

Key Research Accomplishments

1. Establish prostate cancer cell lines stably expressing endostatin and angiostatin

2. Demonstrate the inhibition of endothelial cell proliferation in culture with the angiogenesis inhibitors produced either from direct transduction of the endothelial cells with the endostatin or angiostatin vector or from the culture supernatant harvested from prostate cancer cells transduced with these vectors

3. Demonstrate inhibition of tumor progression *in vivo* with prostate cancer cell lines expressing either endostatin or angiostatin

Reportable Outcomes

1. Construct HIV-based vectors containing the endostatin and angiostatin cDNA

2. Establish PC3 cell lines stably expressing endostatin and angiostatin. These cell lines can be used to evaluate prostate tumor progression *in vivo*.

3. Claudia Kowolik, a Postdoctoral Fellow, received training in HIV vector production, RCL detection and tumor cell implantation onto nude mice. Hardik Modi, a Master student, received training in HIV vector construction and production, RCL detection and tumor cell implantation onto nude mice.

Conclusion

This proposal investigates whether endostatin and angiostatin can be used to treat prostate cancer in animal models. While these two proteins were shown to regress large tumors in animals, systemic administration of such proteins in patients could be problematic because the difficulties to produce them in large quantities and rapid clearance of these proteins from blood. We proposed to use gene therapy approach with HIV-based vectors for the delivery of endostatin and angiostatin genes to treat prostate cancers since such vectors transduce slow-growing cells such as prostate cancer cells more efficiently than other retroviral vectors. Our studies showed that prostate cancer cells were transduced efficiently by the HIV vectors and the angiogenesis inhibitor secreted could inhibit endothelial cell proliferation in culture and tumor formation *in vivo*. However, such inhibition was not complete: tumors continued to progress in grafted nude mice although clear difference in tumor size between control vector-transduced cells and endostatin/angiostatin vector-transduced cells was observed. As all the cell populations were transduced at an efficiency of more than 80% based on GFP expression, the most likely explanation for this observation could be due to inefficient gene expression or protein secretion from the transduced cells. Although the CMV promoter we used to drive gene expression in the HIV vectors is a relatively strong promoter, other promoters such as prostate epithelial cell-specific promoters may be used to drive even more efficient expression of endostatin or angiostatin. For protein secretion, the signal peptide used for the secretion of endostatin or angiostatin can be replaced with a more efficient signal peptide to facilitate protein secretion. These are the future directions for this study. The observation that a combination of endostatin and angiostatin did not inhibit cell proliferation and tumor formation more efficiently than endostatin alone suggests that there is no additive effect with these two factors.

References

1. Yam PY, Li S, Wu J, Hu J, Zaia JA, Yee JK. **Design of HIV vectors for efficient gene delivery into human hematopoietic cells.** *Mol. Ther.* 2002,5:479-484.

2. Parda DS, Thraves PJ, Kuettel MR, Lee MS, Arnstein P, Kaighn ME, Rhim JS, Dritschilo A. **Neoplastic transformation of a human prostate epithelial cell line by the v-Ki-ras oncogene.** *Prostate* 1993,23:91-98.

3. Tanaka T, Cao Y, Folkman J, Fine HA. **Viral vector-targeted antiangiogenic gene therapy utilizing an angiostatin complementary DNA.** *Cancer Res.* 1998,58:3362-3369.

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